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In re application of:	Confirmation No. 1470
Julian Schroeder	Examiner: Collins, Cynthia
Application No.: 09/806,552	Technology Center/Art Unit: 1638
Filed: September 18, 2001	**DECLARATION OF DR. JUDY CALLIS**
For: INHIBITION OF FARNESYLTRANSFERASE ACTIVITY IN PLANTS	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, **Dr. JUDY CALLIS**, hereby declare:

1. I received an A.B. in Biology from Washington University, St. Louis, MO, in 1977, a M.S. in Botany from the University of Illinois, Urbana, IL, in 1981, and a Ph.D. in Biology from Stanford University, Stanford, CA, in 1987. From 1987 to 1989, I was a National Science Foundation Post-doctoral Fellow in the Horticulture Department of the University of Wisconsin-Madison.

2. From 1989 to 1993, I was an Assistant Professor in the University of California Department of Biochemistry & Biophysics. From 1993-2001, I held the position of Associate Professor, in the University of California, Section of Molecular and Cellular Biology. Since 2001, I have been a Professor in the University of California, Davis, Section of Molecular and Cellular Biology. I currently hold the Paul K. and Ruth R. Stumpf Endowed Chair in Plant Biochemistry. I am the author or co-author of some 49 publications in the scientific literature in

the field of plant biology. A copy of my c.v. is attached as Exhibit 1 hereto. I am not an inventor of the captioned application and have no financial interest in it.

3. I am familiar with the production of transgenic plants and with the selection of plants expressing introduced genes, as well as what was known to persons of skill in this art as of September 2001, the filing date of the captioned application.

4. I am aware that the specification discloses the discovery that farnesylation is part of the mechanism for regulating guard cells in closing stomata in plant leaves, and that it is expected that inhibiting farnesylation will therefore result in reducing water loss by transpiration. I am aware that the specification discloses the existence of multiple farnesyltransferase inhibitors and the production of β-glucuronidase ("GUS") in transgenic plant leaves.

5. I have been informed that the Examiner has rejected the claims pending the captioned application as not enabled. I understand that the rejections are based on the argument that persons of skill were not enabled to use the invention without undue experimentation. Specifically, I understand that the Examiner contends that the specification does not provide adequate guidance with respect to which particular inhibitor to express and how to express a chosen inhibitor at concentrations effective to confer a useful phenotype on a transgenic plant. Based on my training and experience in creating transgenic plants and in expressing proteins encoded by genes introduced into those plants, I believe the Examiner's contentions are not only incorrect, but would be considered incorrect by practitioners in this art as of the filing date.

6. As an initial matter, while the Action states that it does not dispute that the level of skill in the art is very high, it argues that this fails to compensate for what it asserts is a lack of guidance with respect to which farnesyltransferase inhibitor to express. In my judgment as a person of skill in the art of making transgenic plants and in expressing the transgenes, I believe this is incorrect. The point of the disclosure of the specification is the discovery of the connection between inhibition of farnesylation and decreased transpiration. Armed with this information and the information already available in the art in September 2001, I believe persons

of skill would be able to make and use transgenic plants which express farnesyltransferase inhibitors, and that would do so at levels effective to inhibit farnesylation in the plants' leaves.

7. As an initial matter, I think the Examiner is wrong that persons of skill were not enabled to make and use the invention because the specification does not suggest which particular farnesyltransferase inhibitor to express. What is important to me, and what would be important to others of skill in the art, is the specification's teaching that inhibiting farnesylation permits decreasing transpiration. Armed with this important information, I believe I would be enabled to make plants with inhibition of farnesyltransferase activity in their guard cells.

8. As the specification observes, a number of farnesyltransferase inhibitors were known as of the filing date. The Action's point seems to be in part that the person of skill would be at a loss as to how to proceed without a teaching of which specific inhibitor to choose. With respect, I would not expect anyone who has created transgenic plants to need more explanation of how to proceed than is provided in the specification. The process would in fact be straightforward. For expression in transgenic plants, persons of skill would select a protein since it is a central dogma of modern biology that genes encode proteins. The choice of the particular inhibitor protein to express would be largely a matter of convenience, based on whether any expression cassettes encoding farnesyltransferase inhibitors could be purchased from laboratory supply houses, borrowed from a colleague, or readily cloned.

9. Further, I am aware that the specification notes that promoters are known that drive expression of constructs specifically in guard cells. Knowledge of how to construct an expression cassette coupling a cell type-specific promoter and a gene of interest is widely available in the art. I also understand that the specification summarizes how to construct a recombinant vector and how to transform a plant to express the transgene. Accordingly, in view of the knowledge in the art and the skill level of its practitioners, I believe the specification contains all the teachings necessary to enable persons of skill to produce plants exhibiting inhibition of farnesyltransferase activity in their guard cells and therefore, according to the teachings of the specification, with decreased transpiration. Certainly, I do not think it was

necessary to provide practitioners with a specific sequence to enable them to make plants expressing a farnesyltransferase inhibitor.

10. I understand that the Action considers there to be no relation between the expression of the reporter protein GUS and the expression of a farnesyltransferase inhibitor in those cells. I further understand that the Action contends that the GUS is "not known or disclosed as being equivalent in function or effect to the protein product [farnesyltransferase] expressed by the claimed plant," and further states "the effect of expressing a nucleic acid encoding a farnesyltransferase inhibitor is unpredictable because expression methods must be specifically adapted in order to achieve a particular desired phenotype, as different levels of protein expression produce different phenotypes, because farnesyltransferase inhibition is dependent on inhibitor concentration and further varies between different types of farnesyltransferase inhibitors, and because compounds that inhibit farnesyltransferase in vitro may be unstable in vivo."

11. Based on my training and experience in expressing recombinant proteins in transgenic plants, I believe the Action's contentions are incorrect, and would be known to be incorrect by persons of skill in this art. The Action is of course correct that GUS is "not known or disclosed as being equivalent in function or effect" to a farnesyltransferase inhibitor. GUS was, however, not supposed to have a function or effect equivalent to that of a farnesyltransferase inhibitor in the study reported -- I and others in the art are aware that GUS is a reporter protein used to show whether an expression cassette results in the expression of a transgene in a desired cellular location and, where appropriate, at a desired point of development. The results reported indicate that the GUS transgene was expressed in the desired cellular location. Thus, the Action's statement that GUS is not shown to be "known or disclosed as being equivalent in function or effect" to a farnesyltransferase inhibitor is a *non sequitor* and ignores what the expression of GUS reported in the specification would evidence to persons of skill in the art.

12. What the expression of GUS reported in the specification shows to me, and would show to others of skill in the art is that the expression of the GUS transgene could be driven in

guard cells, the desired cellular location, by the expression cassette used. To the person of skill in this art, this raises the expectation that other proteins, such as a farnesyltransferase inhibitor, can likewise be expressed in a very specific manner in guard cells by substituting the coding sequence for the inhibitor for the sequence encoding GUS used in the experiment reported.

13. Most coding regions from transgenes will be expressed at levels that can inhibit endogenous activities. There is of course no guarantee that the level of expression will be high in any given plant. Some genes have proven difficult to express in plants, and there are a number of things that could cause problems in expression, such as the presence of a cryptic splice site, instability of RNA transcribed from the expression cassette, causing its rapid degradation, incorrect codon usage resulting in low translation levels, silencing of the transgene so that it does not continue to express, rapid degradation of the expressed protein, resulting in low endogenous levels and, finally, integration of the transgene in a site that inhibits or blocks transcription.

14. Each of these issues can, however, usually be solved by changing nucleic acid sequence of the transgene and/or screening through a number of transgenic plants to find one with high expression. Someone skilled in the art would know that these factors could influence transgene expression and make the necessary alterations. While considerable work might be required (assuming one of the problems mentioned in the preceding paragraph was in fact present), that amount of experimentation is routine in the art when expressing transgenes in plants.

15. Further, there is usually at least some expression of the transgene. And, even if, for example, the protein product was rapidly degraded, the continuing production of fresh protein would likely have some effect on farnesylation in the cell. Thus, and contrary to the Action's analysis, I would expect with a reasonable expectation of success that if I replaced the region coding for GUS with a region coding for a farnesyltransferase inhibitor in the expression cassette used in the study reported in the specification, I would get at least some expression of the farnesyltransferase inhibitor in those cells, and that that expression would have at least some effect on farnesylation in the cell. I do not understand the claims to require any particular amount of inhibition of farnesyltransferase.

16. With respect to the Action's contention that "the effect of expressing a nucleic acid encoding a farnesyltransferase inhibitor is unpredictable because expression methods must be specifically adapted in order to achieve a particular desired phenotype," I respectfully note that the expression of the GUS reporter gene in guard cells already shows to a person of skill in the art that that the expression method was specifically adapted to achieve expression in guard cells.

17. The Action's argument seems to me to prove too much. Persons of skill in this art expect that there are always some variations in expression levels between transgenic plants, even plants made following identical procedures. Even the most detailed teaching in the specification showing the production of a transgenic plant expressing a farnesyltransferase inhibitor with reduced transpiration, therefore, would not permit the practitioner to uniformly produce plants with identical phenotypes. For this reason, practitioners generate a multitude of transgenic plants, select for those that express the transgene, select further for those that express the desired phenotype, and then reproduce those plants asexually or sexually to scale up the number of plants. It is understood by practitioners that it cannot be predicted with any degree of certainty before testing that any particular plant will express a desired transgene at a given level, but it can generally be predicted that a group of transgenic plants will have differences in levels of the transgene and that at least some will express the transgene at phenotypically relevant levels. This degree of experimentation is considered routine in the art. In contrast, under the standard set forth in the Action, no teaching in the specification would be sufficient because it could not teach how to produce plants which always have the same phenotype.

18. As a person of skill in the art of transforming plants to express recombinant proteins, I therefore believe the Action is incorrect in asserting that persons of skill were not enabled to make and use the invention because the specification does not show "how to express a chosen inhibitor at concentrations effective to confer a useful phenotype on a transgenic plant."

19. Finally, I note that the Action contends that the claims are not enabled because farnesyltransferase inhibitors expressed *in vivo* in plants might be unstable or would be degraded too quickly to be effective. There are several problems with the Action's position.

20. First, as noted above, once again, it can generally be predicted that a group of transgenic plants will have differences in levels of the transgene and that at least some will express the transgene at phenotypically relevant levels even if the Action's contentions were otherwise correct.

21. Second, I would expect that for at least some of the plants, the continuing production of fresh farnesyltransferase inhibitor would maintain the effect of inhibiting farnesyltransferase activity below that which would be present in the absence of the inhibitor.

22. Third, I want to address specifically the Action's contention that proteases found in the systemic circulation in mammals are made within the cell and therefore available to degrade farnesyltransferase inhibitors within the cell. This contention ignores the fact that proteins to be secreted from the cell are typically synthesized with a leader sequence that marks the protein for transport to the cell's exterior, where the leader sequence is typically cleaved to produce the active form of the protein. Proteins with these leader sequences are never present in the cell cytosol in an active form. Thus, the fact that there are proteases in the systemic circulation in mammals does not suggest to me as a person of skill that the proteases are also present in the cytosol of the mammalian cells producing them. It further does not suggest to me that such proteases would be present in the cytosol of plant cells, even assuming that plant cells produce such proteases. There is no evidence provided by the Action that proteases would be available within the cell cytosol to degrade a farnesyltransferase inhibitor expressed within the cell. Accordingly, I do not believe that the Action's contentions regarding the possible instability of farnesyltransferase inhibitors or the possible existence of proteases for farnesyltransferase inhibitors within plant cells would affect the ability of the practitioner to reach and sustain phenotypically relevant levels of farnesyltransferase inhibitor expression in transgenic plants.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

August 17, 2005
Date

Judy Callis
Dr. JUDY CALLIS

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Callis, J

Professional Dossier
Dr. Judy Callis

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Education: A.B. (Biology) Washington University, St. Louis, MO, 1977
M.S. (Botany) University of Illinois, Urbana, IL, 1981
Ph.D. (Biology) Stanford University, Stanford, CA, 1987

Professional Positions:

Research Specialist, Department of Plant Pathology, University of Wisconsin-Madison, 1977-1979.

NSF Post-doctoral Fellow, Horticulture Department, University of Wisconsin-Madison, 1987-1989.

Assistant Professor, University of California, Department of Biochemistry & Biophysics, 1989-1993.

Associate Professor, University of California, Section of Molecular and Cellular Biology, 1993-2001.

Professor, University of California, Section of Molecular and Cellular Biology, 2001-present.

Awards and Fellowships:

Awarded, Ruth K. and Paul Stumpf Endowed Chair in Plant Biochemistry 7/2005-7/2010
Elected Fellow, American Association for the Advancement of Science 2002
National Science Foundation Presidential Young Investigator Faculty Award, 1991-1996.
NSF Postdoctoral Fellowship in Plant Biology, 1987-1989.
Awarded ACS Postdoctoral Fellowship, 1986, declined.
McKnight Predoctoral Trainee, 1983-1986.
NIH Predoctoral Trainee, 1981-1983.

Membership in Professional Societies:

American Society for Biochemistry and Molecular Biology
American Society of Plant Physiologists
Genetics Society of America
International Society for Plant Molecular Biologists
The American Society for Cell Biologists

Publications:

1. Callis, J. and Walbot, V. 1977. Determination of the number of ribosomal cistrons in chloroplasts of C3 and C4 plants. In: Nucleic Acids and Protein synthesis in Plants, J. Weil and L. Bogorad (eds.) pp. 137-141.

2. Gurley, W.B., Kemp, J.D., Albert, M.J., Sutton, D.W., and Callis, J. 1979. Transcription of Ti plasmid derived sequences in three octopine-type crown gall tumor lines. Proc. Natl. Acad. of Sci. USA, **76**:2828-2832.

3. Gurley, W.B., Callis, J., and Kemp, J.D. 1979. Crown gall transcription of Ti plasmid-derived sequences. In: Genome Organization and Expression in Plants, C.J. Leaver, (ed.) pp. 481-488.

4. Callis, J. and Ho, D.T.H. 1983. Multiple molecular forms of the gibberellin - induced alpha amylase from the aleurone layers of barley seeds. Archives of Biochemistry and Biophysics, **244**:224-234.

5. Roberts, J.K.M., Callis, J., Wemmer, D., Walbot, V., and Jardetzky, O. 1984. Mechanism of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. Proc. Natl. Acad. Sci. USA, **81**:3379-3383.

6. Roberts, J.K.M., Callis, J., Jardetzky, O.J., Walbot, V., and Freeling, M. 1984. Cytoplasmic acidosis as a determinant of flooding intolerance in plants. Proc. Natl. Acad. Sci. USA, **81**:6029-6033.

7. Fromm, M., Callis, J., Taylor, L. and Walbot, V. 1986. Electroporation of DNA and RNA into plant protoplasts. Methods in Enzymology, **153**:351-366.

8. Callis, J., Fromm, M. and Walbot, V. 1987. Expression of mRNA electroporated into plant and animal cells. Nucl. Acids Res., **15**:5823-5831.

9. Callis, J., Fromm, M. and Walbot, V. 1987. Introns increase gene expression in cultured maize cells. Genes & Development, 1:1183-1200.

10. Callis, J., Fromm, M. and Walbot, V. 1988. Heat inducible expression of a chimeric hsp70cat gene in maize protoplasts. Plant Physiol., **88**:965-968.

11. Vierstra, R.D., Burke, T.B., Callis, J., Hatfield, P., Jabben, M., Shanklin, J., and Sullivan, M. 1988. Characterization of the ubiquitin-dependent proteolytic pathway in higher plants. In *The Ubiquitin System*, Schlesinger, M. and Hersko, A., eds., Cold Spring Harbor Press, Cold Spring Harbor, New York, pp. 119-125.

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13. Roberts, J.K.M., Chang, K., Webster, C., Callis, J. and Walbot, V. 1989. Dependence of ethanolic fermentation, cytoplasmic pH regulation and viability on the activity of alcohol dehydrogenase in hypoxic root tips. Plant Physiol., **89**:1275-127.

14. Callis, J., Pollmann, L., Shanklin, J., Wettern, D., and Vierstra, R.D. 1989. Sequence of a cDNA from *Chlamydomonas renhardii* encoding a ubiquitin 52 amino acid extension protein, Nucl. Acids Res., **17**:8377.

15. Callis, J. and Vierstra, R.D. 1989. Ubiquitin and ubiquitin genes in plants. in Oxford Surveys of Plant Molecular and Cell Biology, **6**:1-30.

16. Callis, J., Raasch, J., and Vierstra, R.D. 1990. The ubiquitin extension proteins of *Arabidopsis thaliana*, structure, localization, and expression of their promoters in transgenic tobacco, J. Biol. Chem., **265**:12486-12493.

17. Hatfield, P.M., Callis, J., and Vierstra, R.D. 1990. Cloning of ubiquitin activating enzyme from wheat and expression of a functional protein in *Escherichia coli*, J. Biol. Chem., **265**:15813-15817.

18. Sullivan, M., Callis, J., and Vierstra, R.D. 1990. HPLC resolution of ubiquitin pathway enzymes from wheat germ, Plant Physiol., **94**:701-716.

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20. Sun, C.-W., and Callis, J. 1993. Recent stable insertion of mitochondrial DNA into an *Arabidopsis* polyubiquitin gene by nonhomologous recombination. The Plant Cell, **5**:97-107.

21. Norris, S., Meyer, S. and Callis, J. 1993. *Arabidopsis* polyubiquitin genes contain introns in the 5' untranslated regions that are quantitative determinants of expression. Plant Molecular Biology, **21**:895-906.

22. Beers, E.P., and Callis, J. 1993. Utility of polyhistidine-tagged ubiquitin in the purification of ubiquitin-protein conjugates and as an affinity ligand for the purification of ubiquitin specific hydrolases. J. Biol. Chem, **268**: 21645-21649.

23. Callis, J. and Bedinger, P. 1994. Developmentally regulated loss of ubiquitin and its conjugates during pollen maturation in maize. Proc. Natl. Acad. Sci. USA **91**: 6074-6077.

24. Callis, J., Carpenter, T., Sun, C.-W., and Vierstra, R.D. 1995. Structure and evolution of genes encoding polyubiquitin and ubiquitin-like proteins in *Arabidopsis thaliana* ecotype Columbia. Genetics **139**: 921-939.

25. Callis, J. 1995. Regulation of Protein Degradation. The Plant Cell. **7**: 845-857.

26. Callis, J. 1997 Regulation of Protein Degradation in Plants Genetic Engineering, **19**: 121-148.

27. Sun, C-W. and Callis, J. 1997 Independent modulation of *Arabidopsis thaliana* polyubiquitin mRNAs in different organs and in response to environmental changes, The Plant Journal, 11: 1017-1027.

28. Chandler, J, McArdle, B., and Callis, J. 1997 *At*UBP3 and *At*UBP4 are two closely related *Arabidopsis thaliana* ubiquitin-specific proteases present in the nucleus, Mol. Gen. Genet., 255: 302-310.

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31. Worley, C.K., Ling, R. and Callis, J. 1998 Engineering *in vivo* instability of firefly luciferase and *Escherichia coli* β-glucuronidase in higher plants using recognition elements from the ubiquitin pathway Plant. Mol. Biol. 37: 337-347.

32. del Pozo, J.C., Timpte, C., Tan, S., Callis, J., and Estelle, M. 1998 AXR1 and the RUB/NEDD8-protein conjugation pathway are required for auxin response in Arabidopsis Science 280: 1760-1763.

33. Rao-Naik, C. dela Cruz, W. Laplaza, J.M. Tan, S. Callis, J. and Fisher, A 1998 The Rub family of ubiquitin-like proteins: Crystal Structure of Arabidopsis RUB1, and expression of multiple RUBs in Arabidopsis, J. Biol. Chem. **273**: 34976-34982.

34. Vierstra, RD, and Callis, J 1999 Polypeptide tags, ubiquitous modifiers for plant protein regulation, Plant Molecular Biology, 41: 435-442.

35. Worley, C.K., Zenser, N, Ramos, J. Rouse, D., Leyser, O., Theologis,A. and Callis, J. 2000 The degradation signal of Aux/IAA proteins is important in auxin signaling, The Plant Journal, 21: 553-562. [Highlighted in Current Opinion in Plant Biology 3: 264 as "of special interest".]

36. Ling, R., Colon, E., Dahmus, M.E., and Callis, J. 2000 Histidine-tagged ubiquitin substitutes for wild-type ubiquitin in yeast and facilitates identification of *in vivo* substrates from yeast and transgenic Arabidopsis, Anal. Biochem. 282: 54-64.

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BUP3, *At*UBP4, and *At*UBP5 *in vivo E. coli* assays and *in vitro*. Archives of Biochem. and Biophysics, 379:198-208.

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41. Ramos, J., Zenser, N., Leyser, O., and Callis, J. 2001. Rapid degradation of Auxin/Indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. The Plant Cell 13: 2349-2360.

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43. Linghu, B., Callis, J., and Goebl, M. 2002 Rub1p processing by Yuh1p is required for wild-type levels of Rub1p conjugation to Cdc53p. Eukaryotic Cell, 1: 491-494.

44. Yoshida, S., Ito, M., Callis, J., Nishida, I. and Watanabe, A. 2002. A delayed leaf senescence mutant is defective in arginyl-tRNA : protein arginyltransferase, a component of the N-end rule pathway in Arabidopsis. Plant J. 32:129-137.

45. Zenser, N., Dreher, K.A. Edwards, S.R., and Callis, J. 2003. Acceleration of Aux/IAA proteolysis is specific for auxin and independent of AXR1 Plant J. 35: 285-294.

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47. Bostick, M. Lochhead, S., Honda, A., Palmer, S. and Callis, J. 2004 RUB1 and RUB2 (Related to Ubiquitin) are redundant, essential, and regulate vegetative growth, auxin signaling, and ethylene production in Arabidopsis. The Plant Cell 16, 2418-2432.

48. Stone, S., Hauksdottir, H., Troy, A., Herschleb, J., Kraft, E., and Callis, J. 2005 Functional analysis of the RING type ubiquitin ligase family of Arabidopsis thaliana L. Plant Physiol., 137: 13-30.

49. Pablo Figueroa, Giuliana Gusmaroli, Giovanna Serino, Jessica Habashi, Ligeng Ma, Yunping Shen, Suhua Feng, Magnolia Bostick, Judy Callis, Hanjo Hellmann, and Xing Wang Deng 2005 Arabidopsis Has Two Redundant Cullin3 Proteins That Are Essential

for Embryo Development and that Interact with RBX1 and BTB Proteins to Form Multisubunit E3 Ubiquitin Ligase Complexes in Vivo. Plant Cell 17, 1180-1195.

50. Callis, J and Ling, R 2005 Preparation, Characterization, and Use of Tagged Ubiquitins. Methods in Enzymol. In press.

Patents and Patent Applications

1. Vierstra, R.D., Hondred, D. and Callis, J. Ubiquitin fusion protein system for protein production in plants. Issued June 30, 1998

2. Callis, J. and Worley, C. Sequences from auxin-induced gene products targeting fusion proteins for degradation patent filed US 5/22/98, Issued, April 24, 2001.

Summary of Research Support:

A. Current Extramural Support:

National Science Foundation IBN-0212659 "Characterization of Aux/IAA Protein Degradation in Higher Plants." $350,000 total costs, 9/1/02-8/30/05 (renewal)

Department of Energy DE-FG03-00ER15056 "The role of Rub (related to ubiquitin) family of proteins in the hormone response pathways" $330,000 total costs, 8/1/03-7/30/06 (renewal)

National Science Foundation MCB-0115870 "Functional Analysis of Ubiqutin E3 Ligases in Arabidopsis." R. Vierstra, UW-Madison, PI. Co-PI with 5 total PIs. $646, 885 total costs to Callis lab, 9/1/01-8/30/05.

B. Past Extramural Support

Department of Energy DE-FG03-00ER15056 "The role of Rub (related to ubiquitin) family of proteins in the auxin response" $300,000 total costs, 7/1/00- 6/30/2003

National Science Foundation IBN-9808791 "Characterization of ubiquitin pathway in higher plants." $440,000 total costs, 9/1/98-8/30/02

NSF IBN-9306759 "Characterization of ubiquitin pathway in higher plants." $583,148 total, 10/93-10/99 (Includes a 1 year no cost extension).

POWRE Supplement to NSF IBN-9306759 "Characterization of ubiquitin pathway in higher plants." $35,000.

ROA Supplement to NSF IBN-9808791 to support the summer research of Dr. Qin Qin Liu, Asst. Professor University of MN-Duluth, $14,981. Summer 1999

NSF DCB 90-05062 "Characterization of ubiquitin pathway in higher plants." $225,000 total, 10/90-10/93.

NSF DCB 91-58453 "Presidential Young Investigator Award". $25,000/year for 5 years (total), 9/91-9/96.

USDA 91-37304-6575 "Characterization of Developmental Regulation of Ubiquitin Conjugation". $95,000 (total), 9/91-9/94.

C. Previous Intramural (UCD) Support:
Faculty Research Grant, "Effect of introns on the expression of genes in higher plants." $2,500 (1990-1991).
Faculty Research Grant, "Effect of introns on the expression of genes in higher plants." $1,950 (1991-1992).

Research Supervisory Role (does not include graduate rotation students):

Post-Doctoral Fellows:

Dr. Sophia Stone (6/03-present)
Dr. Herborg Hauksdottir (04/02-9/03). Lecturer, University of Iceland
Dr. Eric Beers (09/90-12/93) Associate Professor, Virginia Polytechnic Institute
Dr. Mark West (04/92-4/95) Production Enologist, Saintsbury Winery
Dr. Chetna Rao (10/96-8/99) Research Scientist, Medarix, S. San Francisco, CA

Graduate Students:

Plant Biology Graduate Group
Chih-Wen Sun (9/90-3/95) Assistant Professor, Department of Biology, National Taiwan Normal University
Nathan Zenser (6/97-1/02) Research Scienticist, Sigma Chemical Co.
Kate Dreher (6/01-)
Edward Kraft (7/03-)

Biochemistry Graduate Group
Richard Ling (6/92-8/99) Research Scientist, Air Resources Board, Pasadena, CA
Cathy Worley (1/94-12/97) Researcher, Dade Behring, Delaware NJ
Jason Ramos (6/97-6/02) Research Scienticist, Pharmacyclics, S.San Francisco
Magnolia Bostick (5/00-)

Genetics Graduate Group
Jose Miguel Laplaza (6/93-1/01) Post-doctoral Fellow, U. of WI-Madison
Jennifer Chandler (6/93-1/98)
Shirley Tan (6/97-1/99) MS student, Researcher, Roche Molecular Systems, Oakland, CA

Invited Extramural Seminars:

Before 7/93

University of California-Davis, Department of Biochemistry and Biophysics, November, 1988. "Structure and expression of ubiquitin gene family in *Arabidopsis*."

University of Washington, Department of Botany, January 1989. "Structure and expression of ubiquitin gene family in *Arabidopsis*."

University of California-Riverside, Department of Botany and Plant Pathology, March 1989. "Structure and expression of ubiquitin gene family in *Arabidopsis*."

Washington University, Department of Biology, April 1989. "Structure and expression of ubiquitin gene family in *Arabidopsis*."

Sogitol, Inc. Hayward, CA, June 7, 1990. "Ubiquitin genes and conjugation of ubiquitin to protein of higher plants."

University of California-Irvine, Developmental and Cell Biology Seminar Series, May 10, 1990. "Ubiquitin genes and ubiquitin conjugation to proteins in higher plants."

Stanford University, Plant Gene Expression Group, June 20, 1990. "Ubiquitin genes and the ubiquitin pathway in higher plants."

Carnegie Institute of Plant Biology, Stanford CA, February 5, 1992. "Ubiquitin genes and ubiquitin-protein conjugates."

DNA Plant Technology Corporation, March 24, 1992. "Ubiquitin Genes and Finding Ubiquitin Substrates in *Arabidopsis*."

7/93 through 6/95

Cold Spring Harbor Course Guest Lecturer, "Proteolysis and the Ubiquitin Pathway" for the course in *Arabidopsis* Molecular Genetics, July 1993.

University of Arizona, Plant Biology Department, October 4, 1993, "Ubiquitin in Plants: the ubiquitin gene family and searching for in vivo substrates of the ubiquitin pathway".

University of California-Berkeley, Plant Biology Department, April 25, 1994, "Ubiquitin in Plants: the ubiquitin gene family and searching for in vivo substrates of the ubiquitin pathway".

Colorado State University, Cell and Molecular Biology Interdisciplinary Program, Sept. 22, 1994 "The ubiquitin pathway in Arabidopsis: ubiquitin genes and searching for substrates of the pathway".

University of California-Los Angeles Plant Molecular Biology Seminar Series, May 2, 1995, "The ubiquitin pathway: Arabidopsis ubiquitin genes and searching for substrates of the pathway"

7/95 through 6/97

University of California-Riverside, Department of Biochemistry, Oct. 24, 1995 "The ubiquitin pathway: Arabidopsis ubiquitin genes and searching for substrates of the pathway".

Annual Meeting of the Northeast American Society of Plant Physiologists, State University of New York, Plattsburgh, New York, invited speaker, May 3-4, 1996 "The ubiquitin pathway: Arabidopsis ubiquitin genes and searching for substrates of the pathway".

Student invited speaker, DOE Plant Research Lab, Michigan State University, September 17-19 1996.

Invited speaker, Institute of Molecular Biology Academia Sinica, Tapai, Taiwan, February 25, 1997. "The ubiquitin-dependent pathway in higher plants"

Invited speaker, Institute of Botany Academia Sinica, Tapai, Taiwan February 26, 1997. "Determinants of protein instability in higher plants"

Invited Speaker, DowElanco sponsored symposium, "A Symposium on Plant Gene Expression", May 12, 1997 "The Arabidopsis Ubiquitin Gene Family: Expression and Evolution"

Invited speaker, Calgene, Davis-CA May 19, 1997 "The ubiquitin-dependent pathway in higher plants"

7/97 through 6/00

Invited speaker, Interdisciplinary seminar in Plant Sciences, University of Arizona, Tuscon, AZ "Two aspects of auxin signaling in higher plants, degrading Aux/IAA proteins and modifying a component of the ubiquitin pathway" Oct 26, 1998

Invited speaker, Department of Biological Sciences, Georgia State University, Atlanta, GA, The Rub family- proteins related to ubiquitin as modifiers of ubiquitin pathway function? Nov. 6, 1998

Invited speaker, DNA Plant Technology, Oakland CA, April 21, 1999 "Engineering protein degradation in higher plants"

Invited speaker, Department of Biology UC-Santa Barbara, April 29, 1999, "Two aspects of auxin signaling in higher plants, degrading Aux/IAA proteins and modifying a component of the ubiquitin pathway"

Invited speaker, Plant Molecular Biology Consortium, Research Triangle Park, North Carolina (sponsored by UNC-Chapel Hill, NC-State, and Norvartis) May 3-4, 1999. "Two aspects of auxin signaling in higher plants, degrading Aux/IAA proteins and modifying a component of the ubiquitin pathway"

7/00 through 6/03

Department of Biochemistry, University of Wisconsin-Milwaukee, February 26, 2001 "Protein Degradation plays important role in cell signaling: Rub and rapid proteolysis in Arabidopsis in response to the plant hormone auxin"

Plant Biology Seminar Series, Department of Plant Biology and the Plant Research Laboratory, Michigan State University, September 29-30, 2002 "Power of proteolysis in regulating auxin signaling"

University of Michigan-Ann Arbor, Department of Molecular, Cellular, and Developmental Biology, October 2, 2002 ""Regulated Proteolysis in Hormone Pathways in Arabidopsis"

Department of Plant Biology and the USDA Plant Gene Expression Center, November 17, 2002, "The Power of Proteolysis in Regulating Auxin Signaling"

MARC Program, California State Univeristy-Northridge, November 1, 2003, gave research seminar "The Power of Negative Thinking, or How getting rid of proteins is a powerful way to activate or repress gene expression"

At CSU-Northridge, I gave an informal seminar to students about graduate schools in general and graduate opportunities at UC-Davis.

7/03 through 6/04

Invited Speaker, Third International Proteolysis Meeting, Ngoya, Japan, November 10-13, 2003

Invited Speaker, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, November 10, 2003.

Invited Speaker, International Auxin Meeting, Corsica, May 2004

7/04-7/05

Invited Speaker, Carnegie Institution of Plant Biology, October 2004

Invited Speaker, Rice University, Houston TX, Department of Biochemistry and Cell Biology, Dec 2004

Invited Speaker, University of MA-Amherst May 12, 2005
Invited Speaker, University of Minnesota- Minneapolis-St. Paul, June 1-2, 2005

Meeting Participation 00-03

FASEB Meeting on Ubiquitin and Intracellular Proteolysis, Saxton's River VT June 23-30, 2001

Poster- Greta Schrift, Jose Laplaza[2], Judy Callis[2] and Robert E. Cohen[1]
[1]Department of Biochemistry, University of Iowa, Iowa City, IA 52242, and [2]Section of Molecular and Cell Biology, UC Davis, Davis, CA 95616 *"The Yuh1p deubiquitinating enzyme is required for the maturation of Rub1p in* Saccharomyces cerevisiae*"*

Poster, Maggie Bostick, Jose Laplaza, and Judy Callis, *"Rubp1 conjugates in* Saccharomyces cerevisiae".

ASPP Annual Meeting, New Providence, RI, July 2001, J. Callis attended

Invited Speaker to 2010 Workshop, International Arabidopsis Meeting June 2002, Sevilla, Spain
June 30, 2002, "Functional analysis of Ubiquitin Protein (E3) Ligases in Arabidopsis"

International Arabidopsis Meeting June 2002, Sevilla, Spain, June 30, 2002, Maggie Bostick presented poster

Magnolia Bostick, Adria Honda, Michael Warner, Colin Leasure*, Jessica Brown, and Judy Callis. *Transgenic Plants Expressing RNAi for RUB1 Exhibit Defects in Multiple Hormonal Signaling Pathways.*

ASPP Annual Meeting, Denver, CO, July 2002 J. Callis attended, Kate Dreher attended and presented poster.

Cold Spring Harbor Meeting on Intracellular Proteolysis March 03, Maggie Bostick attended and presented poster:

Magnolia Bostick, Adria Honda, Michael Warner, Colin Leasure*, Jessica Brown, and Judy Callis. *Transgenic Plants Expressing RNAi for RUB1 Exhibit Defects in Multiple Hormonal Signaling Pathways.*

Professional Service (see a separate category below for ad hoc grant proposal and manuscript reviews:

7/90 to 6/93
Panel Member, USDA Plant Responses to the Environment (6/91)

Panel Member, NSF Plant Post-Doctoral Fellowships in Plant Biology (3/92)
Panel member, NSF Plant Post-Doctoral Fellowships in Plant Biology (3/93)

7/93 through to 6/95

Member, American Society Plant Physiologists Publications Committee Oct.94-Oct.99 (Begins a 5 year commitment to this committee).

7/95 through to 6/97

Grant Panel Member, USDA Genetic Mechanisms, March 1996
NIH Ad hoc Panel Member, Molecular Cytology, June 1996
NIH Ad hoc Panel Member, Molecular Cytology, Feb 1997
Member, ASPP Publications Committee Oct.95-Oct.97
Member, ASPP Ad Hoc Web Site Committee (96-97)
Reviewer for Chapters in textbook Biochemistry by Garrett and Grisham
Reference for promotion to tenure Dept. of Biochemistry and Cell Biology, Rice University
Reference for promotion to tenure Dept. of Botany and Plant Sciences, University of California-Riverside

7/97 through to 6/99

Instructor, HHMI Biology Institute Summer97
Chair, ASPP Publications Committee Oct 97-Oct. 98
Member, ASPP Publications Committee Oct.98-Oct.99
Member, ASPP Executive Committee Oct 97-Oct. 98
Reviewer for Chapter in new textbook Biochemistry and Molecular Biology of Plants
Member, NSF Grant Review Panel, Integrative Plant Biology (began three year term Spring 99) Served May 1999

7/99 through to 6/00

Member, ASPP Publications Committee Oct.98-Oct.99
Member, NSF Grant Review Panel, Integrative Plant Biology Served Oct. 1999
Reference for promotion to tenure Dept. of Biology, State University of New York-Binghampton

7/00 through to 6/03

Outside Member, Ph.D..Dissertation Committee, Ph.D. in Biochemistry, Elvan Sahid Medical College of Wisconsin March 01
Member, Review Panel for American Society of Plant Biologists Summer Undergraduate Research Awards- reviewed 17 proposals 03/01, 12 proposals 03/03.
Evaluation of scholarly activities for School of Molecular Biosciences, Washington State University, Pullman, WA
Member, NSF Metabolic Biochemistry Review Panel Spring 02
Member, NSF Young Investigator Award Review Panel Spring 03
Member, National Institutes of Health Initial Review Panel CDB-2 Oct 03
Member, International Peer Review Board for Deutsche Forschungsgemeinschaft for Arabidopsis Proteome Project August 01, and June 02
Member, Editorial Board Journal of Plant Physiology- I review papers-about 1/month

Monitoring Editor, Plant Physiology 10/00-10/06- I coordinate the review process- find reviewers and write decision letters. Since becoming a monitoring editor, I have handled 27 papers.
Editor, Faculty of 1000- I write evaluations of recently published papers for an electronic forum
Evaluator for promotion of a member at Academia Sinica, Tapai, Taiwan 9/02
Evaluator for an applicant to the Flanders Interuniversity Institute for Biotechnology, Sp03

Ad hoc reviewer 00-03

Molecular Biology of the Cell 10/00, 9/01
FEBS Letters 9/01
PNAS Fall 00
The Plant Journal- 1-2 a year
Plant Cell Reports-1
Planta-1
Plant Science-1
J. Biological Chemistry-1
Department of Energy Ad Hoc reviewer- Fall 00- 2 proposals
National Science Foundation Fall 00-2 proposal, Spring 01-2 proposals, Fall 02- 3 proposals, Fall 03-3 proposals, Spring 03-4 proposals
USDA Spring 01-3 proposals, Spring 03-1 proposal
Netherlands Organization for Scientific Research- review of research proposal
Reviewer for Austrian Der Wissenschaftsfonds START Program application
Reviewer for the Israel Science Foundation, proposal Sp03

The Plant Cell, Vol. 8, 2381–2394, December 1996 © 1996 American Society of Plant Physiologists

Protein Farnesyltransferase in Plants: Molecular Characterization and Involvement in Cell Cycle Control

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Farnesylation is required for membrane targeting, protein–protein interactions, and the biological activity of key regulatory proteins, such as Ras small GTPases and protein kinases in a wide range of eukaryotes. In this report, we describe the molecular identification of a plant protein farnesyltransferase (FTase) and evidence for its role in the control of the cell cycle in plants. A pea gene encoding a homolog of the FTase β subunit was previously cloned using a polymerase chain reaction–based strategy. A similar approach was used to clone a pea gene encoding a homolog of the FTase α subunit. The biochemical function of the pea FTase homologs was demonstrated by the reconstitution of FTase enzyme activity using FTase fusion proteins coexpressed in *Escherichia coli*. RNA gel blot analyses showed that levels of FTase mRNAs are generally higher in tissues, such as those of nodules, that are active in cell division. The relationship of FTase to cell division was further analyzed during the growth of suspension-cultured tobacco BY-2 cells. A biphasic fluctuation of FTase enzyme activity preceded corresponding changes in mitotic activity at the early log phase of cell growth. Moreover, manumycin, a specific inhibitor of FTase, was effective in inhibiting mitosis and growth in these cells. Using synchronized BY-2 cells, manumycin completely blocked mitosis when added at the early S phase but not when added at the G_2 phase. These data suggest that FTase is required for the plant cell cycle, perhaps by modulating the progression through the S phase and the transition from G_1 to the S phase.

INTRODUCTION

Isoprenylation is required for membrane association and cellular activity of many eukaryotic regulatory proteins. This post-translational modification involves covalent attachment of an isoprenyl moiety (either 15-carbon farnesyl or 20-carbon geranylgeranyl) to the cysteine residue of a short motif (CXXX) at the C terminus of proteins (Farnsworth et al., 1990; Glomset et al., 1990; Maltese, 1990; Rilling et al., 1990; Hancock and Marshall, 1993; Casey, 1994). The isoprenyl moiety also plays a critical role in the specific interaction of isoprenylated proteins with other signaling proteins (Fukada et al., 1990; Marshall, 1993; Kisselev et al., 1994; Piorfini et al., 1994). Three distinct enzymes catalyzing isoprenylation reactions have been identified in yeast and mammals, each modifying proteins with unique C-terminal motifs (Kohl et al., 1991; Moores et al., 1991; Kinsella and Maltese, 1992). Protein farnesyltransferase (FTase) modifies proteins with CAAX (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid except for leucine and phenylalanine). In contrast, protein geranylgeranyltransferase I (GGTase I) requires CAAL (where L is leucine) at the C terminus of substrate proteins (Casey et al., 1989, 1991; Vorburger et al., 1989; Khosravi-Far et al., 1991; Moores et al., 1991; Reiss et al., 1991b). GGTase II recognizes proteins with the C-terminal sequence CC, CCXX, or CXC plus an unidentified internal sequence (Farnsworth et al., 1991; Khosravi-Far et al., 1991; Moores et al., 1991; Kinsella and Maltese, 1992). Among the three enzymes, the structure and function of FTase is most extensively studied in yeast and mammalian systems.

FTases are composed of two nonidentical subunits, α and β (Chen et al., 1991a, 1991b; He et al., 1991; Reiss et al., 1991a), and genes encoding both subunits have been cloned from yeast and mammals (Chen et al., 1991a, 1991b; He et al., 1991). The two subunits form a stable complex, and both are required for catalytic activity (Chen et al., 1991a, 1991b; He et al., 1991; Reiss et al., 1991a). Each subunit contains five conserved internal sequence repeats that are thought to be critical for enzymatic activity (Boguski, 1992; Andres et al., 1993). The function of the α subunit is not clear, although it has been suggested that it binds isoprenyl pyrophosphate (Reiss et al., 1991a, 1992). The β subunit binds the substrate protein in a Zn^{2+}-dependent manner (Reiss et al., 1991a, 1992). Nonetheless, the mechanisms underlying subunit interaction and regulation of enzyme activity remain essentially unknown.

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Proteins known to be farnesylated include the Ras small GTPases, fungal mating factors, the γ subunit of heterotrimeric G proteins, prelamins A and B, protein kinases, and molecular chaperones (Hancock et al., 1989; Inglese et al., 1992b; Zhu et al., 1993; Sinensky et al., 1994; Ong et al., 1995). Thus, protein farnesylation is involved in the modulation of many important processes, including signal transduction, cell cycle control, protein export, and development (Sepp-Lorenzino et al., 1991; Cox et al., 1992; Inglese et al., 1992a, 1992b; Kato et al., 1992; Hancock and Marshall, 1993; Marshall, 1993; Kisselev et al., 1994; Hara and Han, 1995; Kauffmann et al., 1995; Scheer and Gierschik, 1995). One of the most important functions of FTase appears to be its involvement in the control of cell cycle and mitogenic signaling. Differential farnesylation of Ras at the G_1 phase and of nuclear lamins at the early S phase was shown in synchronized human cells (Sepp-Lorenzino et al., 1991). FTase mRNA and enzyme activity levels are higher in Ras-induced human skin carcinomas than in normal skin cells (Khan et al., 1996). Moreover, farnesylation is required for the function of Ras in the mitogenic signaling that controls the G_0/G_1-to-S transition in animal systems (Cox et al., 1992; Kato et al., 1992; Bokoch and Der, 1993; Itho et al., 1993; Kauffmann et al., 1995; McCormick, 1995). Recently, an oncogenic human protein tyrosine phosphatase thought to be involved in mitogenic signaling was also found to be farnesylated (Cates et al., 1996). The FTase α subunit binds to and is phosphorylated by mammalian transforming growth factor-β (TGF-β) receptors, suggesting the intriguing possibility that FTase is a bona fide signaling enzyme (Kawabata et al., 1995; Wang et al., 1996).

Farnesylation may also be involved in the control of the cell cycle by modulating the targeting and activity of nuclear lamins (Moir et al., 1995). In *Xenopus* oocytes, farnesylated lamin B has been implicated in the regulation of DNA replication (Firmbach-Kraft and Stick, 1993, 1995; Moir et al., 1995). Lamin B binds to proliferating cell nuclear antigen and matrix attachment regions in replication foci, and lamin B–depleted *Xenopus* nuclei are unable to replicate DNA (reviewed in Moir et al., 1995). In human cells, lamins are differentially farnesylated in the S phase, consistent with the notion that farnesylation of lamin B plays an important role in the regulation of DNA replication (Sepp-Lorenzino et al., 1991). However, direct evidence for the role of farnesylation in DNA replication is lacking.

Recent studies have demonstrated the existence of FTase in plants and suggest potential roles in plant growth regulation (Randall et al., 1993; Zhu et al., 1993; Morehead et al., 1995; Parmryd et al., 1995; Cutler et al., 1996). Genes encoding the plant homolog of the FTase β subunit have been cloned from pea and *Arabidopsis* (Yang et al., 1993; Cutler et al., 1996). Putative substrate proteins have been identified, including a functional homolog of DnaJ from *Atriplex nummularia* and a family of novel proteins from soybean (Zhu et al., 1993; Biermann et al., 1994).

We are interested in understanding the regulation and physiological function of FTase in plants. In this study, we cloned the pea gene encoding the α subunit homolog (PsFTa) and demonstrated functional identity of FTase by reconstituting FTase activity from the pea α and β (PsFTb) subunits coexpressed in *Escherichia coli*. Further analyses of FTase enzyme activity and FTase inhibitor-induced inhibition of cell division strongly support a role for FTase in plant cell cycle control.

RESULTS

Cloning and Characterization of a Pea Gene Encoding a Homolog of the FTase α Subunit

Two degenerate oligonucleotides (FTa3 and FTa4) corresponding to repeats 2 and 3 of the rat FTase α subunit (Chen et al., 1991a; Andres et al., 1993) were used to amplify a product of ~130 bp from a pea root tip cDNA library. Sequence analysis revealed that this fragment encodes a deduced polypeptide of 44 amino acids with strong sequence similarity to the FTase α subunits from rat and yeast (61% identical to rat and 39% identical to yeast) (Chen et al., 1991a; He et al., 1991). DNA gel blot hybridization analysis showed that this fragment hybridized with a single HindIII fragment in pea genomic DNA (data not shown).

The 132-bp polymerase chain reaction (PCR) fragment was used to screen the pea root tip cDNA library, and three clones (designated *PsFTa1*, *PsFTa2*, and *PsFTa3*) containing the longest inserts were analyzed further. As shown in Figure 1, *PsFTa1* and *PsFTa2* have identical nucleotide sequences, except that *PsFTa1* (1138 bp) is shorter at both ends than is *PsFTa2* (1285 bp). *PsFTa1* starts with nucleotide +14 and ends with the polyadenylated tail at nucleotide 1151, whereas *PsFTa2* starts with nucleotide +6 and ends with the polyadenylated tail at nucleotide 1291. The difference in length at the 3′ untranslated sequence is most likely due to use of alternative polyadenylation sites. The third clone, *PsFTa3*, is a chimera between a 354-bp sequence overlapping 5′ regions of *PsFTa1* and *PsFTa2* and a promiscuous sequence. This chimeric sequence is probably the result of recombination during in vivo excision of plasmid cDNA from phage. Nonetheless, *PsFTa3* is 5 bp longer at the 5′ end than is *PsFTa2* and includes a possible ATG initiation codon that establishes an open reading frame encoding a predicted polypeptide of 333 amino acids.

To determine whether this ATG is indeed a translation initiation codon, we used an EcoRI fragment at the 5′ end of the clone *PsFTa1* to isolate four additional clones from a pea genomic library. All four clones are identical in sequence and contain a stop codon at nucleotides −18 to −16 upstream of the first ATG codon in *PsFTa3* (Figure 1). Furthermore, there is no obvious consensus splicing acceptor sequence ($[T/C]_nNC/TAG$) between this stop codon and the first ATG (Mount, 1982), and there are several pairs of AG dinucleotides between the stop and ATG codons. These AG pairs are rarely seen in the −15 through −5 region of an acceptor (Seif et al., 1979). Sequences surrounding ATG (GAACATGGC) conform to the plant consensus translation initiation sequence AAC-

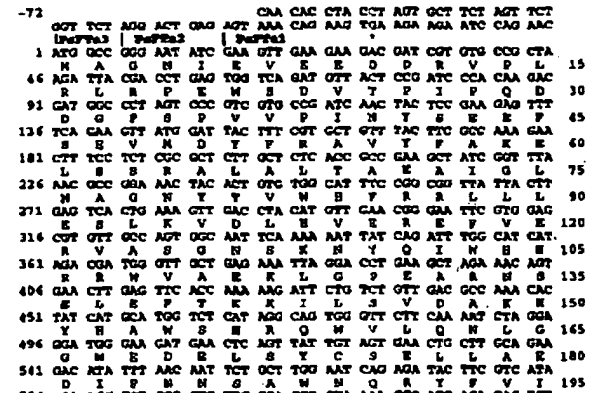

Figure 1. Nucleotide and Predicted Amino Acid Sequences of the cDNA Encoding the Pea FTase α Subunit.

Shown is a DNA sequence composite of three cDNA clones (*PsFTa1*, *PsFTa2*, and *PsFTa3*) and a genomic clone. The starting position of each cDNA clone is indicated. Nucleotide position +1 indicates the putative translation initiation codon. The nucleotide sequence is numbered at left; the amino acid sequence is numbered at right. The first in-frame stop codon upstream of the putative initiation codon and the stop codon at the end of the open reading frame are marked with asterisks. The nucleotide sequence has been submitted to GenBank with accession number U63298.

AATGGC (Lutcke et al., 1987). In addition, the longest cDNA clone is close to the length of the *PsFTa* transcript (~1.4 kb) as revealed by RNA gel blot hybridization (see below). It is therefore very likely that the 999 nucleotides represent the entire coding region of the pea FTase α subunit.

The PsFTa polypeptide predicted from the open reading frame has an estimated molecular mass of 38.5 kD. As shown in Figure 2, PsFTa exhibits strong sequence similarity to the FTase α subunits from rat and yeast (Chen et al., 1991a; He et al., 1991). PsFTa contains the five tandem sequence repeats present in all FTase α subunits from other organisms (Boguski, 1992; Andres et al., 1993; Feng and Kung, 1993). The positions of four conserved residues, glutamate, asparagine, tryptophane, and arginine, are invariant in all five repeats, except for the glutamate residue in the first repeat. Like the β subunit homolog (Yang et al., 1993), PsFTa exhibits greater overall sequence similarity to the mammalian α subunit (37% identity to rat FTase α subunit) than to the yeast α subunit (25% identity). Both N- and C-terminal regions are less conserved. Like the yeast FTase α subunit, PsFTa is ~50 amino acids shorter at the N terminus than is the mammalian FTase α subunit. Only 12 of 99 residues at the C terminus of PsFTa are identical to the yeast or mammalian α subunit.

To assess *PsFTa* gene copy number, we performed genomic DNA gel blot hybridization analysis. As shown in Figure 3, a single EcoRV or HindIII fragment was detected under both low- and high-stringency conditions. The probe cross-hybridized with two EcoRI fragments because the *PsFTa1* cDNA contains an EcoRI site. These results suggest that PsFTa is encoded by a single gene in pea.

FTase Activity Reconstitution from *E. coli*-Expressed Fusion Proteins

To demonstrate that the pea homologs of FTase subunits (PsFTa and PsFTb) form a functional FTase enzyme, we attempted to reconstitute FTase activity by using PsFTa and PsFTb fusion proteins expressed in *E. coli*. *PsFTa* and *PsFTb* coding sequences were each fused to a glutathione *S*-transferase (*GST*) gene in a pGEX-based vector, and novel polypeptides of expected molecular sizes were detected (Figure 4A). Each GST fusion protein was affinity-purified using glutathione-conjugated Sepharose, and a mixture of an equal amount of each protein was used for FTase activity assays. The fusion proteins resulted in no significant FTase activity (data not shown). Similar phenomena were observed with independently derived subunits of fungal and mammalian FTase (He et al., 1991). This was presumably the result of incorrect folding or assembly of independently expressed fusion proteins.

To circumvent this problem, we constructed a bicistronic plasmid (pGEX-*FTa/b* shown in Figure 5) to direct coexpression of the two polypeptides in the same *E. coli* cell. The expression of the two fusion proteins was confirmed by protein gel analysis, as shown in Figure 4B. Extracts from this *E. coli* strain were used for the FTase activity assay, using tritiated farnesyl pyrophosphate (^{3}H-FPP) and a yeast recombinant Ras protein (Ras-CAIM) as substrates (Randall et al., 1993). As shown in Figure 4C, ^{3}H-FPP was incorporated into Ras-CAIM in the reaction containing protein extracts from cells coexpressing PsFTa and PsFTb fusion proteins or the yeast FTase subunits (He et al., 1991). The labeled Ras proteins appeared as doublet bands, probably as a result of protein degradation or two different protein conformations. No ^{3}H-FPP was incorporated into Ras-CAIM in the reactions containing PsFTa or PsFTb fusion protein alone. The recombinant protein Ras-SVLS, which cannot be farnesylated by the fungal or mammalian FTase, was not labeled (Figure 4C). These results demonstrate that PsFTa and PsFTb comprise a functional pea FTase.

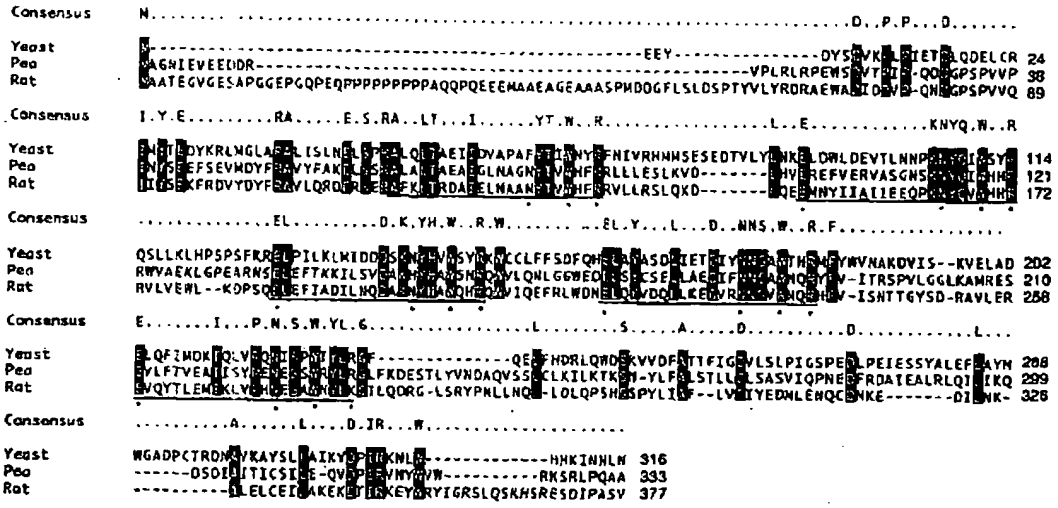

Figure 2. Comparison of Amino Acid Sequences for the FTase α Subunits from Pea, Rat, and Yeast.

The deduced amino acid sequences of the FTase α subunit from rat (Chen et al., 1991a) and yeast (He et al., 1991) were aligned to *PsFTa* with Megaline (DNASTAR, Inc., Madison, WI). Numbers at right indicate residue positions of the predicted amino acid sequence. Consensus residues are indicated by black rectangles. Gaps introduced into the alignments are indicated by dashes. Five internal amino acid sequence repeats are underlined. Four invariable residues (E, N, R, and W) within the repeats are marked with asterisks.

Under our assay conditions (2 hr at 30°C), ^{3}H-FPP showed weak binding to a 15-kD *E. coli* protein, regardless of which fusion proteins were expressed in *E. coli*, indicating that the labeling of this *E. coli* protein was not the result of FTase activity (see Figure 4C). In addition, ^{3}H-FPP appeared to bind to proteins corresponding to the size of GST–PsFTa (62 kD) or the yeast α subunit (38 kD) whenever these proteins were present. This result supports the hypothesis that the FTase α subunit plays a role in binding isoprenyl substrates (Reiss et al., 1991a, 1992).

Coordinate and Differential Regulation of Gene Expression for FTase Subunits during Plant Development

To gain clues to the physiological function of FTase in plants, we studied expression patterns for genes encoding the pea FTase α and β subunits by using RNA gel blot hybridization analyses. As shown in Figure 6A, a *PsFTa* transcript of ~1400 nucleotides was detected in most parts of pea plants. Transcript levels were highest in nodules in which plant cells presumably divide actively. Moderate levels of *PsFTa* transcripts were found in roots and floral buds, whereas transcript levels were very low or barely detectable in other parts, such as open flowers, leaves, and stems. Because the roots used for RNA isolation were infected with *Rhizobium* spp and may have contained microscopic nodules, these tissues might have contributed significantly to the transcript levels detected in roots. Rehybridization of the RNA blot with a probe for the pea 23S RNA gene (Yang and Watson, 1993) showed that most samples contained equivalent amounts of RNA; slightly lower amounts of RNA were loaded for young leaves, large nodules, and stems.

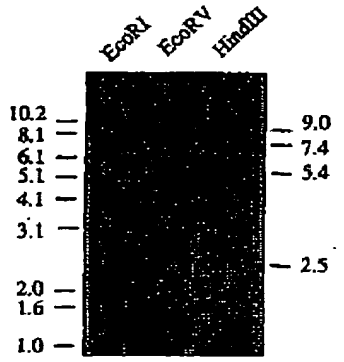

Figure 3. Genomic DNA Gel Blot Analysis of *PsFTa*.

Five micrograms of pea genomic DNA was digested with the indicated restriction enzymes and hybridized with the *PsFTa1* cDNA, as described in the text. Molecular length markers are indicated at left in kilobases. Numbers at right indicate lengths in kilobases of genomic DNA fragments that hybridized with the probe.

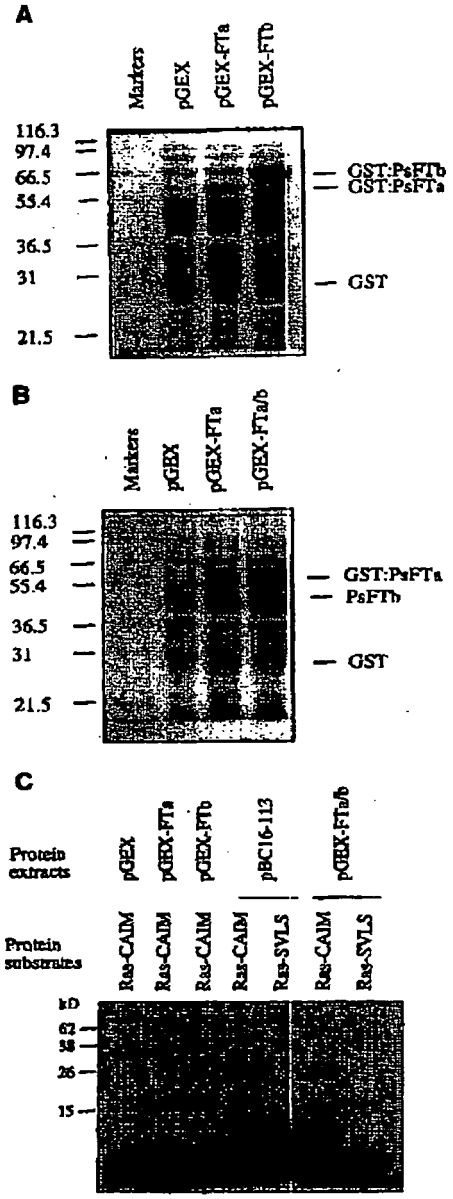

Figure 4. Expression of Pea FTase Subunits in *E. coli* and Reconstitution of FTase Enzyme Activity.

(A) Expression of pea FTase α and β subunits as GST fusions in *E. coli*. Five milliliters of isopropyl β-D-thiogalactopyranoside–induced *E. coli* cells expressing GST (pGEX) or GST fusion proteins (GST:PsFTa and GST:PsFTb) were centrifuged and resuspended in 300 μL of protein gel loading buffer. Ten microliters of protein extracts was separated on a 10% SDS–polyacrylamide gel, and proteins were visualized by staining gels with Coomassie Brilliant Blue R 250.

(B) Coexpression of pea FTase subunit proteins in *E. coli* by using the bicistronic plasmid shown in Figure 5. Protein extracts from *E. coli* cells expressing GST (pGEX) and GST–PsFTa fusion (pGEX-FTa) or coexpressing GST–PsFTa fusion and PsFTb (pGEX-FTa/b) were separated on a 10% SDS–polyacrylamide gel and stained with Coomassie blue.

(C) FTase enzyme assays for subunit proteins expressed in *E. coli*. Enzyme assays were conducted using ^{3}H-FPP and recombinant Ras proteins (Ras–CAIM and Ras–SVLS), as described in text. Designations are as given in (A) and (B): pBC16-113, the plasmid expressing the yeast FTase α and β subunits (Caplin and Marshall, 1995). The 62-kD band is the GST–PsFTa fusion, the 38-kD band is the yeast α subunit, the 26-kD band is the yeast Ras recombinant protein, and the 15-kD band is a nonspecific FPP binding protein from *E. coli*. In (A) and (B), numbers at left indicate protein molecular mass markers in kilodaltons.

The expression pattern of *PsFTb* was similar to that of *PsFTa* in all parts of pea plants, except for floral buds (Figure 6B). In floral buds, *PsFTa* transcript levels were relatively high, whereas the *PsFTb* transcript was barely detectable. The *PsFTb* transcript detected in roots consistently showed somewhat reduced mobility; the molecular basis of this observation remains to be determined.

These results indicate that although the expression of *PsFTa* and *PsFTb* genes is coordinately regulated in most tissues, there is differential regulation for these two genes in certain parts of pea plants. Furthermore, these results show that FTase gene expression is highly regulated during the plant developmental processes. In general, greater amounts of FTase mRNA are found in tissues of nodules and floral buds undergoing active cell division, suggesting a potential role for plant FTase in cell cycle regulation analogous to that found for the mammalian FTase.

Changes in FTase Activity Are Correlated with Cell Division in Suspension-Cultured BY-2 Cells

The relationship of FTase activity to cell division was directly assessed in the well-characterized tobacco BY-2 suspension-cultured cell system (Nagata et al., 1992). BY-2 cells were harvested at different times after the transfer of stationary cells to fresh medium, and cell fresh weight, mitotic index, and FTase enzyme activity were determined.

No significant increase in cell fresh weight was observed until 3 days after subculture, and growth continued through day 7 (Figure 7A). The mitotic activity (assessed as a percentage of cells with condensed chromatin) followed an apparent biphasic change (Figure 7B). A rapid increase in the mitotic index occurred 24 hr after subculture. After reaching the first peak within 36 hr, the mitotic index leveled off by 48 hr. This was followed by a second major peak between days 3 and 4

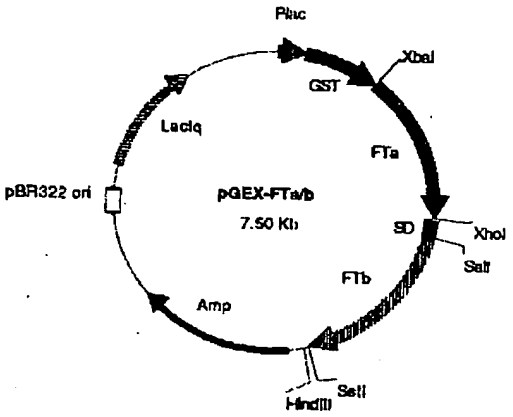

Figure 5. Map of the Bicistronic Plasmid pGEX-FTa/b.

A bicistronic gene that encodes the GST–PsFTa fusion protein and PsFTb was constructed into the expression vector pGEX-KG, as described in text. Amp, ampicillin resistance gene; FTa, *PsFTa1* cDNA; FTb, *PsFTb* coding sequence; LacIq, *Lac* repressor gene; ori, origin of replication; Ptac, *lac* promoter; SD, Shine–Dalgarno sequence.

and a steady decline to the low activity of stationary cells by day 7.

The kinetics of FTase enzyme activity also followed a biphasic fluctuation that precedes the fluctuation of mitotic activity (Figure 7C). Enzyme activity increased within 6 hr after subculture and reached the first peak by 12 hr, before any observed increase in mitotic activity. Enzyme activity declined between 24 and 36 hr, which is coincident with the first rapid increase in mitotic index. FTase activity reached a maximum by day 3 and decreased by day 4, again preceding changes in mitotic activity. These results provide further evidence for the potential involvement of FTase in regulation of the plant cell cycle.

A Competitive Inhibitor of FTase Blocks Cell Cycle Progression in BY-2 Cells

The role of farnesylation in cell cycle control was directly tested using manumycin, a specific inhibitor of protein FTase (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Hara and Han, 1995; Nagase et al., 1996). This cell-permeable inhibitor was shown to be selective for FTase, but not for a related enzyme GGTase I or enzymes involved in the biosynthesis of isoprenoid intermediates, and thus has been widely used to study the function of FTase in yeast and animals (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Hara and Han, 1995; Nagase et al., 1996).

To test whether manumycin also functions as a selective FTase inhibitor in plant cells, we investigated the effect of manumycin on in vivo protein isoprenylation in BY-2 cells. Cells were initially treated with mevinolin (a hydroxymethylglutaryl CoA reductase inhibitor) to block the endogenous production of isoprenoids (e.g., FPP, geranylgeranyl pyrophosphate [GGPP]); these cells were then incubated with ^{3}H-FPP or ^{3}H-GGPP in the presence of manumycin. As shown in Figure 8, manumycin at a concentration as low as 2.5 μM completely blocked in vivo protein farnesylation but not geranylgeranylation. These results are consistent with the effect of manumycin in animal and yeast systems, in which it was shown that IC_{50} (concentration that results in 50% inhibition) for the inhibition of FTase by manumycin is ~2.5 μM (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Nagase et al., 1996).

To study its effect on cell division, we added manumycin to suspension-cultured BY-2 cells at various times after subculture in fresh medium, and mitotic indices were determined every 24 hr. As shown in Figure 9A, manumycin strongly inhibited cell division when added 1 or 2 days after subculture. Manumycin treatment on day 4, by which time FTase activity had dropped and mitotic activity had leveled off in untreated cultures (see Figure 7), had no significant effect on cell division. Because of the time intervals (36-hr time point was not taken), the biphasic mitotic activity was not observed in these experiments.

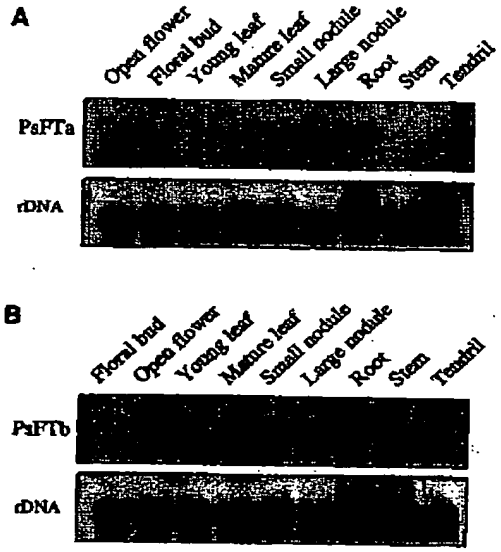

Figure 6. RNA Gel Blot Analysis of Pea FTase Transcript Levels.

Thirty micrograms of total RNA from various organs or tissues of pea plants was separated on an agarose gel, transferred to nylon membranes, and hybridized with the ^{32}P-labeled *PsFTa* and *PsFTb* cDNA probes. Similar specific activity was used for the two probes, and both RNA blots were exposed to x-ray film for 1 week. Each blot was rehybridized to the pea 23S rDNA probe.
(A) Hybridization with the *PsFTa* cDNA probe.
(B) Hybridization with the *PsFTb* cDNA probe.

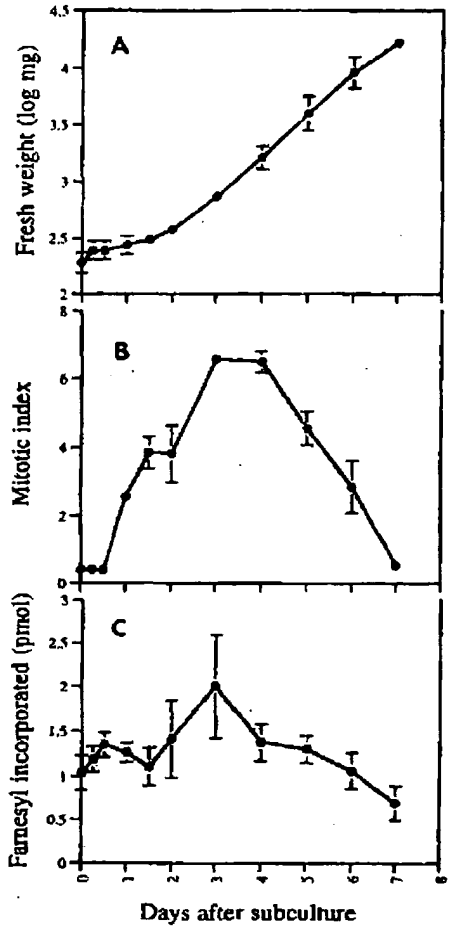

Figure 7. Changes in FTase Enzyme Activity and Mitotic Indices during Tobacco BY-2 Cell Suspension Culture.

Seven-day-old cells were transferred to fresh Murashige and Skoog medium. At various times after transfer, cells were harvested, weighed, and aliquoted for FTase assays, and the mitotic index was determined as described in the text. The data shown are the mean of two replicates. The standard error is indicated where the value exceeded the size of the symbols used.

(A) Fresh weights of BY-2 cells from 50 mL of suspension-cultured cells collected on filter paper.

(B) Mitotic index determined from scoring 1000 cells stained with 1% orcein and calculated as the percentage of cells with condensed chromatin.

(C) FTase enzyme activity presented as total amount of ^{3}H-FPP incorporation into Ras proteins in one standard reaction, as described in the text.

To assess whether the effect of manumycin on cell division simply resulted from cell growth inhibition, we determined fresh weights at the end of the culture cycle (day 7). As shown in Figure 9B, cell fresh weights were significantly lower in cultures treated with manumycin on day 1 and day 2, and thus were correlated with the inhibition of cell division. However, cell fresh weights for cultures treated with manumycin on day 4 were comparable to untreated cells. Because logarithmic cell growth continues throughout the culture cycle (see Figure 7A), these results indicate that manumycin does not have a direct effect on cell growth. The addition of farnesyl pyrophosphate to manumycin-treated cells rescued cell division to a large extent, providing further evidence for the specific effect of manumycin on tobacco FTase (data not shown).

To analyze further the effect of manumycin on cell cycle progression, we used BY-2 cells that were synchronized by the addition of aphidicolin, a specific inhibitor of DNA polymerase. Stationary phase BY-2 cells were transferred to fresh medium and arrested in the early S phase with aphidicolin (Nagata et al., 1992). Aphidicolin inhibition was released by washing the cells, and manumycin was added at the time of aphidicolin release (0 hr) or at the G_2 phase (6 hr after aphidicolin release; Nagata et al., 1992). As shown in Figure 10, manumycin treatment at the early S phase (0 hr) completely blocked mitotic activity. In contrast, treatment at the G_2 phase (6 hr) did not inhibit mitosis. These results suggest that manumycin blocks cell cycle progression through the S phase but has no effect on the G_2-to-M transition.

DISCUSSION

In a previous study, we cloned a gene encoding a pea homolog of the FTase β subunit (PsFTb) (Yang et al., 1993). In this

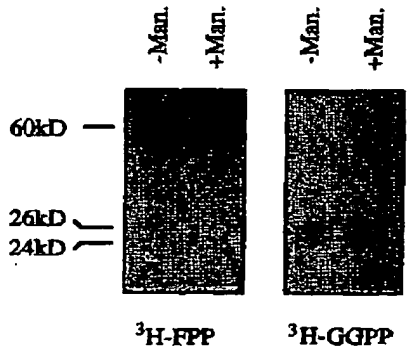

Figure 8. Inhibition of In Vivo Protein Farnesylation by Manumycin.

Mevinolin-treated BY-2 cells were incubated with ^{3}H-FPP or ^{3}H-GGPP in the absence (–) or presence (+) of 2.5 μM manumycin (Man.). Cell-free protein extracts were separated by SDS-PAGE, and farnesylated proteins were visualized by fluorography, as described in the text. Numbers at left indicate protein molecular mass markers in kilodaltons.

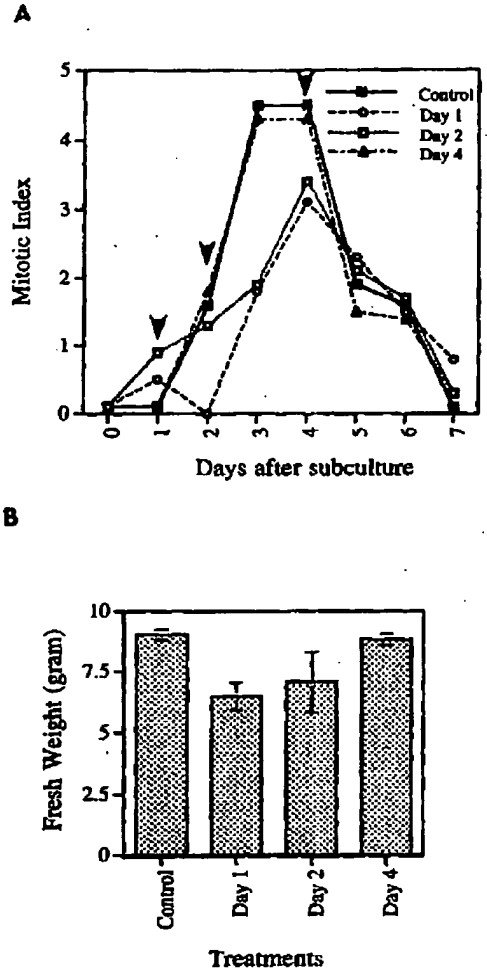

Figure 9. Inhibition of Cell Division by Manumycin in BY-2 Cell Suspension Cultures.

Two hundred microliters of 7-day-old cells was transferred to 20 mL of fresh medium. Manumycin was added to cultures at the time indicated by arrowheads, and the mitotic index was determined every 24 hr, as described in Figure 7. Fresh weights were measured on day 7. Control indicates no addition of manumycin. Data are the mean from two independent experiments. For mitotic indices, standard errors were <10% in all samples; for fresh weights, standard errors are indicated.
(A) Mitotic indices.
(B) Fresh weights.

study, we describe the cloning of *PsFTa*. Sequence analysis reveals that the deduced amino acid sequence of PsFTa has strong similarity to the FTase α subunit from mammals and yeast and contains the five direct tandem repeats found in the yeast and mammalian α subunits. These conserved repeats were shown to be essential for enzyme catalytic activity of the mammalian FTase (Andres et al., 1993). Although the pea FTase subunits show regions that are distinct from previously characterized FTases, our data definitively demonstrate their molecular identities based on reconstitution of FTase activity in *E. coli* coexpressing the *PsFTa* and *PsFTb* gene products.

Our data on the expression of *PsFTa* and *PsFTb* genes and the regulation of FTase activity support the hypothesis that FTase is involved in the control of cell cycle progression in plants. First, RNA gel blot hybridization analyses indicated that the expression of FTase genes is associated with tissues undergoing rapid cell division. In addition, there is a close correlation between changes in FTase activity and mitotic activity in suspension-cultured tobacco cells. In our studies, both the enzyme activity and mitotic index followed an apparent biphasic pattern of fluctuation during the growth of suspension-cultured BY-2 cells (Figure 7). The first peak may reflect the synchronization of a small population of BY-2 cells after the transfer of stationary cells to fresh medium, whereas the second major peak is likely to represent nonsynchronous division for the majority of cells. Because the degree of BY-2 cell synchronization is highly dependent upon growth conditions,

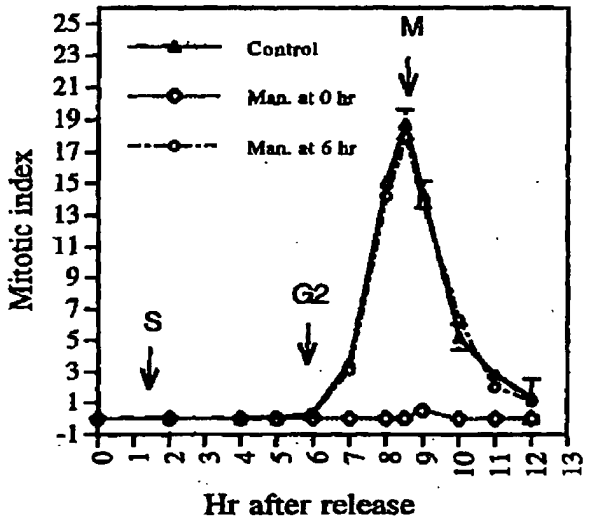

Figure 10. Inhibition of Cell Cycle Progression by Manumycin in Synchronized Cells.

Seven-day-old cells were transferred to fresh medium and synchronized to the early S phase by using aphidicolin, as described in the text. Manumycin (Man.; 2.5 μM) was added at the time of aphidicolin release or 6 hr after the release, and the mitotic index was determined as described in Figure 6. The data shown are the mean of two replicates. The standard error is indicated where the value exceeded the size of the symbols used. The S, G_2, and M phases were estimated to be ~1.5, 6, and 8.5 hr after aphidicolin release, according to Nagata et al. (1992).

subtle differences in our conditions could explain why the biphasic fluctuation of the mitotic index was not observed in previous investigations (Nagata et al., 1992). Interestingly, our data demonstrate that the changes in FTase activity precede the changes in mitotic activity in both phases by at least 6 hr. Because there is a lag period of ~6 hr between the S and M phases of the cell cycle in synchronized BY-2 cells (Nagata et al., 1992), these results are consistent with farnesylation being involved in the early events of the cell cycle such as the G_0/G_1-to-S transition.

The role for FTase in cell cycle control in plants is directly supported by experiments involving the FTase competitive inhibitor manumycin. Manumycin strongly inhibited cell division only when added 1 or 2 days after transfer to fresh medium (Figure 9A). Manumycin had no effect on cell growth or division when added on day 4, by which time FTase activity dropped and mitotic activity started to decline while logarithmic cell growth continued (see Figure 7). This indicates that the inhibition of cell division by manumycin is not related to cell growth per se. Moreover, manumycin blocks cell cycle progression in synchronized BY-2 cells when added at early S phase but not at G_2. These results agree with those of Morehead et al. (1995), who used general isoprenylation inhibitors. The treatment of suspension-cultured tobacco BY-2 cells with the hydroxymethylglutaryl coA reductase inhibitor mevinolin or the protein isoprenylation inhibitor perillyl alcohol inhibited growth of suspension-cultured BY-2 cells, but only when it was added within the first 2 days after transfer to fresh medium (Morehead et al., 1995).

Several lines of evidence strongly suggest that manumycin blocks cell cycle progression in BY-2 cells by specifically inhibiting FTase activity. First, we showed that manumycin at a concentration as low as 2.5 μM (IC_{50} value for fungal and mammalian FTase) effectively inhibited protein farnesylation but not geranylgeranylation in BY-2 cells. Second, the inhibition of cell division by manumycin can be rescued by exogenously supplied FPP. Third, the timing of sensitivity to manumycin treatments in suspension-cultured BY-2 cells is similar to that for mevinolin and perillyl alcohol (Morehead et al., 1995), suggesting that manumycin is most likely to affect only the processes that involve protein isoprenylation. Moreover, manumycin was shown to have no effects on the biosynthesis of isoprenoid products in mammalian cells (Nagase et al., 1996). Finally, the timing of effective inhibition of cell division by manumycin is in accord with the change in FTase activity and mitotic activity in suspension-cultured cells, further supporting the selectivity of this inhibitor for FTase.

Our data imply a role for FTase in the regulation of S phase progression and the G_1/G_0-to-S transition in plants. In mammalian cells, specific farnesylation of several proteins has been linked to cell cycle progression: lamins are farnesylated between the G_1 and S phases and Ras is farnesylated in the G_1 phase (Sepp-Lorenzino et al., 1991). Nuclear lamins have been implicated in DNA replication in *Xenopus* oocytes (Moir et al., 1995). Plant proteins related to mammalian nuclear lamins have been described previously (Frederick et al., 1992; McNulty and Saunders, 1992; Tong et al., 1993); however, their farnesylation and role in DNA replication remain to be determined. In mammalian cells, farnesylation is required for the activation of Ras GTPases involved in the mitogenic signaling that controls the G_0/G_1-to-S transition (Cox et al., 1992; McCormick, 1995). A Ras homolog has not been identified in plants to date. Nonetheless, incorporation of ^{3}H-FPP into tobacco proteins of similar molecular sizes as mammalian lamins (55 to 60 kD) and Ras proteins (20 to 26 kD) supports the notion that farnesylated lamin and Ras homologs are present in plants (see Figure 8). Identification of proteins that are differentially farnesylated at the G_1 and S phases will further define the role of protein farnesylation in the control of the cell cycle in plants.

Apart from cell cycle control, plant FTase may be involved in other aspects of plant growth and development. Homologs of the chaperone DnaJ and heavy metal binding proteins were found to be farnesylated in plants, suggesting a potential role for FTase in stress responses (Zhu et al., 1993; Randall et al., 1996). Recently, several Arabidopsis mutants that exhibit hypersensitivity to abscisic acid (ABA) were identified (Cutler et al., 1996). Surprisingly, one of these mutants was characterized as a T-DNA insertion mutation in the gene encoding an FTase β subunit homolog. This finding suggests a critical role for FTase in ABA signaling and ABA-mediated growth regulation.

PsFTa mRNA but not *PsFTb* mRNA accumulates to significant levels in floral buds, although the accumulation of these two transcripts is coordinately regulated in most pea organs or tissues (see Figure 6). There is evidence that both FTase and GGTase I share the same α subunit in yeast and mammalian cells (Seabra et al., 1991). If this also holds true for plant isoprenyltransferases, the more abundant floral *PsFTa* transcript may be required for GGTase I activity. It is also possible that there are multiple isoforms of the FTase β subunit that share the same PsFTa. This notion is supported by our DNA gel blot hybridization analysis indicating that the pea genome contains a small gene family encoding the β subunit (D. Qian and Z. Yang, unpublished results), whereas PsFTa is encoded by a single gene (Figure 3). Additional research will address whether plants indeed have multiple FTase isozymes and whether each plays a distinct role in modulating one of the potential FTase-dependent processes described above.

The mechanism for the regulation of FTase activity is likely to involve divergent regions of the subunit proteins. The pea FTase subunits have distinct structural features. PsFTb contains a region of ~50 amino acids near the C terminus, which is absent in the yeast and rat counterparts (Yang et al., 1993). Both the pea and yeast FTase α subunits (He et al., 1991; see Figure 2) lack an N-terminal proline-rich domain of ~50 amino acids in length that is present in the rat FTase α subunit (Chen et al., 1991a). This domain is not essential for enzyme catalytic activity (Andres et al., 1993). The C-terminal regions among the plant, yeast, and mammalian α subunits are also quite variable (see Figure 2). Recent studies show that the mammalian FTase α subunit binds to and is phosphorylated by a TGF-β receptor, suggesting that FTase may be directly regulated by other signaling proteins (Kawabata et al., 1995; Wang et al.,

1996). Perhaps, the unique domains in the plant FTase subunits play a role in the interaction with plant-specific signaling proteins, analogous to the TGF-β receptor interaction. Hence, elucidating the biochemical function of these domains may provide novel insight into the role of FTase regulation in signal transduction.

METHODS

Plant Materials

Pea (*Pisum sativum* cv Alaska) seedlings were germinated and grown on moist paper towels in darkness for 4 days before being transferred to a growth chamber. After 1 day in the growth chamber under constant light at 22°C, leaves were harvested for DNA isolation. For RNA extraction, different parts, including small nodules (<2 mm), large nodules (>2 mm), roots without visible nodules, fully expanded mature leaves, rapidly expanding young leaves, tendrils, stems, floral buds, and mature open flowers, were harvested from mature plants grown in a growth room at 22°C under constant white light.

Polymerase Chain Reaction Amplification and cDNA Cloning and Sequencing

Two degenerate oligonucleotides, FTa3 (5'-CAA/GCCIAAA/GAAC/TTAC/TCAA/GGTTTGG-3') and FTa4 (5'-CCAIGCG/ATGG/ATAG/ATTT/CTTIGCA/GTC-3'), corresponding to amino acid residues 162 to 169 (QPKNYQVW) and 196 to 203 (DAKNYHAW), respectively, of the rat farnesyltransferase (FTase) α subunit (Chen et al., 1991a), were used as primers for polymerase chain reactions (PCR). Phage DNA from a pea root tip cDNA library constructed in λZAP (T. Jacob, University of Illinois, Urbana-Champaign) was isolated by using the method of Sambrook et al. (1989). PCR amplifications were performed in a 100-mL reaction mixture containing 100 ng of phage DNA, 400 pmol of each primer, and 5 units of Taq DNA polymerase (Perkin-Elmer Cetus). Thirty-five amplification cycles were conducted at 94°C for 1 min (denaturation), 45°C for 30 sec (annealing), and 72°C for 30 sec (synthesis). Ends of amplified PCR products were filled in using *Escherichia coli* DNA polymerase I and phosphorylated using T4 polynucleotide kinase and ATP. Blunt-ended fragments were cloned into the SmaI site of pBluescript SK− (Stratagene) and sequenced using Sequenase Version 2.0 (United States Biochemical). Three clones were sequenced and found to contain identical 132-bp fragments, which encode a 44–amino acid polypeptide exhibiting strong sequence similarity to both mammalian and yeast α subunits.

The 132-bp fragment, labeled with ^{32}P-dCTP by using a random primer labeling system (Gibco BRL), was used to screen the pea root tip cDNA library. Duplicate filters were hybridized in a solution containing 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 10 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's solution is 0.4% [w/v] Ficoll, 0.4% [w/v] polyvinylpyrrolidone 0.4% [w/v] BSA), 40% formamide, and 100 μg/mL sonicated single-stranded salmon sperm DNA at 42°C. Of 2 × 10^6 plaques screened, >30 positive clones were identified. Selected clones were excised in vivo, according to the instructions of the manufacturer (Stratagene). Three clones containing inserts of ~1.3 kb were sequenced on both strands, as described above. Because all of these cDNA clones appear to lack 5' ends, genomic clones were isolated by screening ~2 × 10^6 plaques of a pea genomic library from S. Gantt (University of Minnesota, St. Paul) by using an EcoRI fragment from the 5' end of the cDNA clone, *PsFTa1* (see Figure 1). Five positive clones were identified, subcloned into pBluescript SK−, and sequenced using a primer complementary to the 5' end of the *PsFTa1* cDNA sequence (Figure 1, nucleotides +55 to 75).

DNA and RNA Gel Blot Hybridization Analyses

Genomic DNA was isolated according to the protocol of Dellaporta et al. (1983). Total RNA was isolated as described previously (Yang et al., 1993). Five micrograms of pea genomic DNA was digested with EcoRI, EcoRV, or HindIII, separated on a 0.9% agarose gel by electrophoresis, and blotted onto a GeneScreen membrane (Du Pont). The membrane was hybridized with the ^{32}P-labeled *PsFT1* cDNA clone under high-stringency conditions at 65°C in 5% SDS, 0.25 M NaH_2PO_4. Filters were washed twice in 7% SDS and 0.1 M NaH_2PO_4 at 65°C for 30 min each and then twice in 1% SDS and 0.1 M NaH_2PO_4 at 65°C for 30 min each. For RNA gel blot hybridization, 30 μg of total RNA from different pea tissues was denatured and separated on 1.0% agarose–formaldehyde gels, according to Sambrook et al. (1989). RNA was transferred onto a Nytran membrane (Schleicher & Schuell) and hybridized with the ^{32}P-labeled *PsFTa1* cDNA or *PsFTb* coding sequence in 50% formamide, 6 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/mL of sonicated denatured salmon sperm DNA at 42°C. RNA blots were washed at 65°C in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate) and 0.5% SDS for 30 min and exposed to x-ray film for 5 to 7 days. To standardize the RNA loading, membranes were stripped and rehybridized to a probe encoding the pea 23S rRNA (Yang and Watson, 1993).

Expression of α and β Subunit Fusion Proteins in *E. coli* and Enzyme Activity Reconstitution

The pea homologs of FTase α and β subunits (PsFTa and PsFTb) were expressed as glutathione *S*-transferase (GST) fusion proteins in *E. coli* by using a pGEX derivative pGEX-KG (Guan and Dixon, 1991). To construct the GST–PsFTb fusion, we amplified the *PsFTb* coding sequence (Yang et al., 1993) by PCR and cloned it into the SmaI site of pBluescript SK−. The insert was excised from the resulting plasmid pBS-FTb by using EcoRI and SstI and subcloned into pGEX-KG to create pGEX-FTb. For the GST–PsFTa fusion, the cDNA clone *PsFTa1* (see Figure 1) was excised from pBluescript SK− with XbaI and XhoI and cloned into corresponding sites of pGEX-KG, resulting in pGEX-FTa. *E. coli* DH5α containing pGEX-FTa or pGEX-FTb was grown at 37°C to an OD_{600} of 1.0. To induce the expression of fusion proteins, we added isopropyl β-D-thiogalactopyranoside to a final concentration of 1.0 mM, and incubation of cultures was continued for an additional 3 hr. Soluble proteins were isolated and fusion proteins purified on glutathione–Sepharose (Pharmacia). Equal amounts of fusion proteins were mixed and used for FTase assays, as described below.

To coexpress the PsFTa and PsFTb fusion proteins in the same *E. coli* strain, we constructed a bicistronic cassette containing both *PsFTa* and *PsFTb* genes (Figure 5). An oligonucleotide containing a Shine–Dalgarno sequence, a translation initiation codon, and restriction sites (XhoI, SalI, SstI, and HindIII) (top strand, 5'-TCGAGGAGAACAGATGGTCGACTTTGAGCTCA-3'; bottom strand, 5'-AGCTTGAGCTCAAAGTCGACCATCTGTTCTCC-3') was inserted into XhoI and HindIII sites of pGEX-FTa. The *PsFTb* coding sequence was excised from pBS-FTb

and subcloned into SalI and SstI sites within the oligonucleotide downstream of the translation start codon. The resulting bicistronic plasmid, designated pGEX-FTa/b, was transformed into *E. coli* DH5α. A single colony was inoculated into 5 mL of Luria-Bertani liquid medium. Bacterial cells were cultured at 37°C to an OD_{600} of 1.0. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.33 mM, and cultures were incubated for an additional 4 hr at room temperature.

The cells were harvested by centrifugation and resuspended in 250 μL of lysis buffer (50 mM Hepes, pH 7.5, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin, 0.1 mM leupeptin, and 1 mg/50 mL lysozyme). The lysates were stored at −70°C. Immediately before the assay, frozen cell lysates were thawed on ice, 10 μg/mL DNase I was added, and lysates were centrifuged at 50,000*g* for 15 min using a TL100 ultracentrifuge (Beckman, Fullerton, CA). Protein extracts were used for FTase assays as described below.

Determination of Growth and Mitotic Index of Tobacco BY-2 Cells

Suspension-cultured tobacco BY-2 cells were incubated by shaking at room temperature in Murashige and Skoog liquid medium containing 4.3 g/L Murashige and Skoog salts (Sigma), 100 mg/L inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D, 255 mg/L KH_2PO_4, pH 5.0, and 30 g/L sucrose. Routine cultures were maintained by transferring 1 mL of 7-day-old cells to 50 mL of fresh medium in a 250-mL flask every 7 days. For the kinetic study of FTase activity and mitotic activity, cells were harvested at different time points after the transfer to fresh medium. Cells were collected by filtering through P8 filter paper (Fisher Scientific, Pittsburgh, PA), and fresh weights were measured. Cells from each time point were aliquoted for FTase assay and mitotic index score. Cells for FTase assays were frozen in liquid nitrogen and kept at −70°C until use.

The mitotic index was scored at the time of harvest. To determine the mitotic index, we stained cells with a 1% orcein solution in 45% acetic acid. One thousand stained cells were examined for each sample, and the examination was repeated at least twice. The mitotic index is calculated as the percentage of cells with condensed chromatin (Katsuta et al., 1990).

In Vitro FTase Enzyme Assay

To determine FTase enzyme activity in suspension-cultured BY-2 cells, we prepared cell-free extracts by grinding frozen BY-2 cells in a prechilled mortar with ice-cold extraction buffer (250 mM mannitol, 50 mM Hepes, 3 mM EGTA, 1 mM EDTA, pH 7.5, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL pepstatin, 5 μg/mL chymostatin). Homogenates were centrifuged at 50,000*g* for 15 min using a TL100 ultracentrifuge to remove cell debris. The protein concentration was determined by the Bradford method (Bio-Rad).

In vitro FTase activity was assayed as described previously (Randall et al., 1993). Briefly, a typical reaction (136 μL) contained 50 mM $MgCl_2$, 50 μM $ZnCl_2$, 5 mM DTT, 50 mM Hepes, pH 7.5, 450 μg of cell-free protein extract, and 10 μg of Ras–CAIM (where A is alanine, I is isoleucine, and M is methionine) or Ras–SVLS (where S is serine, V is valine, and L is leucine) recombinant proteins. Recombinant Ras–CAIM and Ras–SVLS were kindly provided by D.N. Crowell (Indiana University–Purdue University at Indianapolis; Randall et al., 1993). This mixture was prequilibrated at 30°C for 2 min before the addition of 2.5 μCi of tritiated farnesyl pyrophosphate (^{3}H-FPP) (60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO; or Du Pont–New England Nuclear, Boston, MA). The reaction mixture was incubated at 30°C for 40 min and terminated with 1 M HCl in 100% ethanol. Precipitated proteins were collected and washed with 15 mL of 100% ethanol on G6 glass fiber filters (Fisher Scientific). Air-dried filters were counted by a liquid scintillation counter. To confirm the incorporation of ^{3}H-FPP into Ras substrates, we separated a 10-μL aliquot of reaction mixtures on a 14% SDS–polyacrylamide gel, and labeled proteins were detected by fluorography. Gels were fixed in isopropanol–water–acetic acid (25:65:10 [v/v/v]) for 30 min, incubated in Amplify fluorographic reagent (Amersham, Arlington Heights, IL) for 40 min, and dried onto filter paper before being exposed to x-ray film for 1 week at −70°C.

To determine FTase activity of *E. coli*–expressed FTase fusion proteins, we added either 18 μg of purified fusion proteins or 490 μg of extracts of *E. coli* cells expressing different fusion proteins as described above to reaction mixtures. As negative controls, extracts from *E. coli* strains containing pGEX, pGEX-FTa, or pGEX-FTb were used. For positive controls, an *E. coli* strain expressing the yeast FTase α and β subunits was used (He et al., 1991). This strain was provided by M. Marshall (Indiana University Medical Center, Indianapolis; Caplin and Marshall, 1995) with the permission of S. Powers (University of Dentistry and Medicine of New Jersey, Piscataway). The incorporation of ^{3}H-FPP into the recombinant Ras protein was determined by the SDS-PAGE analysis and fluorography as described above.

Synchronization of BY-2 Cells

Cells were synchronized essentially as described by Nagata et al. (1992). Seven-day-old cells were diluted 50-fold in fresh Murashige and Skoog media containing aphidicolin (5 mg/L) and cultured with shaking for 24 hr. Aphidicolin inhibition was released by washing cells in fresh media at least three times. Washed cells were incubated in fresh medium with shaking, and an aliquot of cells was taken every 30 min or 1 hr for the determination of mitotic indices, as described above.

Manumycin Treatments

Manumycin was obtained from M. Hara (Kyowa Hakko Kogyo Co., Tokyo, Japan). A stock solution of 10 mM in DMSO was stored in darkness. At various times after subculture or synchronization, manumycin was added to BY-2 suspension cultures to a final concentration of 2.5 μM.

In Vivo Protein Farnesylation Assay

A stock solution (10 mM) of mevinolin (a gift to C.L. Cramer from Merck Sharp & Dohme Research Laboratories, Rahway, NJ) was prepared as described by Bach and Lichtenthaler (1982). Mevinolin was added to 2-day-old BY-2 cells to a final concentration of 10 μM. After 16 hr, treated cells were allowed to settle. An aliquot of 35 μL of settled cells was resuspended in an equal volume of fresh medium containing 5.0 μM manumycin and 0.5 μCi radioactive isoprenyl substrates (^{3}H-FPP, 60 Ci/mmol or ^{3}H geranylgeranyl pyrophosphate [^{3}H-GGPP], 19.3 Ci/mmol). Cells were incubated for an additional 4 hr before being washed with 5 mL of Murashige and Skoog medium. Washed cells were homogenized in 30 μL of 1 × protein loading buffer (50 mM Tris-

Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Cell lysates were centrifuged in a microcentrifuge, and 20 μL of the supernatants was subjected to electrophoresis and fluorography, as described above.

ACKNOWLEDGMENTS

We thank Drs. Dring N. Crowell and Brenda J. Biermann for their assistance with FTase enzyme assays and for their generous gift of Ras-CAIM and Ras-SVLS recombinant proteins. We also thank Dr. Mark Marshall for the plasmid expressing the yeast FTase subunits, Dr. Scott Power for his permission to use the yeast FTase genes, Dr. Thomas Jacob for the pea root tip cDNA library, Dr. Mitsunobu Hara for a gift sample of manumycin, Dr. Yalai Wang for pea RNA isolation, and Dr. Junko Katsuta for her assistance with mitotic index determination. This work was supported by a grant from the National Science Foundation to Z.Y. and C.C. (No. MCB9496224).

Received August 23, 1996; accepted October 23, 1996.

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Purified yeast protein farnesyltransferase is structurally and functionally similar to its mammalian counterpart.

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Protein farnesyltransferase (FTase) catalyses the addition of a farnesyl group to a cysteine within the so-called 'CAAX box' at the C-terminus of various proteins. In the present paper we report purification of Saccharomyces cerevisiae FTase to near-homogeneity. This was accomplished by constructing a yeast strain overproducing FTase approx. 100-fold. The purified enzyme was a heterodimer of approx. 90 kDa and consisted of 43 kDa and 34 kDa subunits. The 43 kDa subunit was shown to be the product of the DPR1 gene by using antibody raised against baculovirus-produced DPR1 polypeptide. The purified enzyme required Mg2+, showed a pH optimum of 7.8 and was most active at 50 degrees C. The Km values for farnesyl pyrophosphate and GST-CIIS (glutathione S-transferase fused to the C-terminal 12 amino acids of yeast RAS2 protein), KmFpp and KmGST CIIS, were 8.1 and 5.1 microM respectively. The enzyme was capable of farnesylating GST-CIIL (the same as GST-CIIS, except that the C-terminal serine is changed to leucine), a substrate protein for the enzyme geranylgeranyltransferase, although with a higher apparent Km than for GST-CIIS. Like its mammalian counterpart, yeast FTase activity was inhibited by peptides containing the C-terminal CAAX sequence (that is, one where C = cysteine, A = aliphatic amino acid and X = any amino acid). These results provide direct evidence for the idea that the yeast and mammalian FTases are structurally and functionally very similar.

PMID: 8424764 [PubMed - indexed for MEDLINE]

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Identification of spinach farnesyl protein transferase. Dithiothreitol as an acceptor in vitro.

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Spinach seedlings were found to contain farnesyl protein transferase. The enzyme is activated by Zn2+, but not by Mg2+. The pH optimum is approximately 7.0 and maximal activity is obtained at 40-45 degrees C. The apparent Km for the farnesyl diphosphate substrate is 7 microM. Western blotting of soluble proteins with an antiserum raised against mammalian farnesyl protein transferase demonstrated a specific cross-reactivity with the spinach enzyme. The antiserum preferentially recognises the beta-subunit of the heterodimeric farnesyl protein transferase, and the corresponding spinach polypeptide has a molecular mass of 42 kDa on SDS/PAGE. The enzyme can employ dithiothreitol as an acceptor for the farnesyl moiety and catalyses the formation of a thioether linkage between these substrates. On the basis of this discovery, a new method was developed utilising the hydrophobicity of the reaction product, and its interaction with poly (propylene). During in vivo labelling, the plants took up dithiothreitol, which inhibited the incorporation of [3H]mevalonate metabolites into proteins, indicating that dithiothreitol might be isoprenylated in vivo as well as in vitro. However, isoprenylation of some proteins remains unaffected by dithiothreitol suggesting the existence of different isoprenylation mechanisms. Thus, it is demonstrated that plants possess farnesyl protein transferase, which resembles its mammalian and yeast homologues.

PMID: 8575428 [PubMed - indexed for MEDLINE]